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Protein Synthesis in Isolated Castor Bean Mitochondria Is Stimulated by Cyanide[1]

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ABSTRACT

Cyanide added to isolated castor bean (*Ricinus communis* L.) mitochondria supplemented with ATP and succinate (or NADH) significantly enhanced the rate and extent of organellar protein synthesis. Cyanide stimulated mitochondrial protein synthesis in a dose-dependent manner with an optimum stimulation of over twofold at 1 millimolar cyanide. At this concentration of cyanide, the mitochondrial respiratory activity, in the presence of succinate (or NADH) and ADP was inhibited by 90%. The stimulatory effect of cyanide on mitochondrial translation was reflected in the increased synthesis of all the proteins synthesized within the organelle. Preliminary evidence indicates a role for the alternative, salicylhydroxamic acid-sensitive, oxidase in the cyanide stimulation of protein synthesis.

Castor bean mitochondria contain a complete genetic system capable of expressing a limited number of proteins for the development of the organelle into a functional state (6, 9). We have reported on the characteristics of protein synthesis in mitochondria isolated from germinated castor beans (12, 13). They showed remarkable similarities with those of other higher plants in their energy requirements, sensitivity to various inhibitors and the pattern of polypeptides synthesized (4, 10, 15).

Cyanide, a classical inhibitor of respiration, should inhibit castor bean mitochondrial protein synthesis by reacting with cytochrome oxidase, hence preventing the formation of ATP (21). However, we have observed that cyanide, paradoxically, stimulated the protein synthetic capacity of the isolated mitochondria from castor beans. This phenomenon was seen when the mitochondria were supplied with ATP or an ATP-regenerating system together with succinate or NADH. The conditions for this stimulatory effect of cyanide on the mitochondrial protein synthesis have been investigated and show that it has definable substrate requirements, dose-dependency upon cyanide and persists when respiration is almost completely shut-down.

MATERIALS AND METHODS

Mitochondria

Mitochondria were prepared under stringent aseptic conditions from 4 d germinated castor beans (*Ricinus communis* L.) grown in the dark at 30° C in Levington compost (Fisons, UK). After removing the testa, the endosperms were washed with water and then surface sterilized in 10% (w/v) sodium hypochlorite with constant agitation for 15 min at 4° C. All subsequent procedures were performed at 0° C to 4° C. After washing extensively in several changes of sterile distilled water, the seeds were chopped and homogenized (1 g endosperms per 4 ml of 0.3 M mannitol, 30 mM Mops/KOH [pH 7.4], 1 mM EDTA, 0.1% (w/v) BSA, 0.05% (w/v) L-cysteine, 0.01% (w/v) D-cycloserine) using a Polytron (Kinematica, Switzerland) PT35 2/M probe set at speed 6 for 2 to 3 s. The homogenate was gently squeezed through four layers of muslin cloth, and a crude mitochondrial pellet obtained by differential centrifugation by the method of Bonner (2). The pellet was suspended in resuspension buffer (homogenization buffer without cysteine) and fractionated by isopycnic centrifugation in 30% (v/v) Percoll at 110,000*g* for 30 min; two distinct bands at the densities of 1.05 g/mL and 1.07 g/mL were resolved. The upper band was carefully harvested, diluted fivefold with resuspension buffer and recovered by centrifugation at 25,000*g* for 15 min. The purified mitochondrial pellet was gently homogenized in resuspension buffer at protein concentration of 10 mg/mL.

Translation

Protein synthesis by freshly-isolated mitochondria was performed using a modification of the procedure of Leaver *et al.* (15). Protein synthesis was carried out in 50 μL final volume containing 0.25 M mannitol, 90 mM KCl, 10 mM KH_2PO_4, 7.5 mM $MgCl_2$, 1 to 3 mM each of 19 L-amino acids (11), 2 mM DTT, 20 μCi [^{35}S]methionine (1350 Ci/mmol, Amersham International, UK), various components for production of energy, described below, and mitochondria at a final protein concentration of 1.5 mg/ml. The pH of the final mixture was 7.4. Energy to sustain protein synthesis was generated by (a) respiratory-linked phosphorylation on addition of 5 mM Na-succinate plus 2.8 mM ADP, and/or (b) 2.2 mM NADH plus 2.8 mM ADP, and/or (c) by an ATP regenerating system consisting of 5 mM ATP, 6 mM creatine phosphate and 0.4 unit of creatine phosphokinase (150 units/mg protein), or (d) 5 mM Na-succinate and 5 mM ATP. The precise conditions are indicated in the text. Synthesis was initiated on addition of mitochondria and temperature shift from 0° to 20° C. Freshly prepared KCN, dissolved in water, was added, where indicated in the text, to the translation system after the addition of mitochondria. The mixture was aerated by agita-

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tion at 100 cycles/min and the incubation was allowed to progress for 1 h.

Protein Synthesis

This was monitored in triplicate volumes of 2 μL of the translation mixtures by measuring the amount of [^{35}S]methionine incorporated into synthesized protein (16).

Respiration

Mitochondrial respiration was monitored polarographically using Rank oxygen electrode (Rank Brothers, Bottisham, Cambridge. UK). Freshly isolated mitochondria (1.5 mg protein) were incubated in 3 mL reaction buffer composed of 0.25 M mannitol, 15 mM KCl, 5 mM $MgCl_2$, 5 mM K_2HPO_4 (pH 7.2) in a cell thermostatically controlled at 30° C. After establishing a steady state of O_2 consumption with succinate (10 mM) or NADH (5 mM), ADP (0.25 mM) was added and the rate of respiration measured (5).

RESULTS AND DISCUSSION

Freshly isolated castor bean mitochondria when provided with succinate (10 mM) and ADP (0.25 mM) gave respiration rates of 70 ± 5 nmol O_2/min/mg protein; the respiratory control was 2.4 and the ADP/O ratio was 2.1. By these criteria the isolated mitochondria were assessed to be intact and functional (17).

The mitochondria incorporated [^{35}S]methionine into TCA-insoluble products in the presence of exogenous energy sources supplied in the form of either ATP or succinate (NADH) supplemented with ADP (Table I). It is improbable that the protein synthesis was due to the presence of contaminating bacteria since no detectable radiolabel incorporation was observed with acetate as the sole exogenous carbon source (15). Protein synthesis was undetectable when the mitochondrial suspension was supplemented either with ADP or with oxidizable substrates (succinate, NADH) (Table I). These findings indicate that isolated castor bean mitochondria are deficient in adenine nucleotides or lack endogenous energy resource(s) even to support the synthesis of minute quantities of protein detected by the highly sensitive radiolabelling method employed here. However, significant protein synthesis was observed (a) when the oxidizable substrates were supplemented with either ADP or ATP, or (b) with ATP, provided either directly or via an ATP-regenerating system. Thus, to sustain protein synthesis, the isolated mitochondria have to either import external ATP or generate energy by processes linked to respiration. The data presented in Table I summarize these observations. Maximum incorporation of amino acids was observed in the presence of succinate and ADP.

As expected, the addition of cyanide (1 mM) to the isolated mitochondria resulted in substantial inhibition of the organellar protein synthesis when the energy was furnished by oxidation of either succinate or NADH in the presence of ADP (Table I). The small but significant stimulatory effect of cyanide on the translation system, supplemented with either ATP or an ATP regenerating, was unexpected. These results

Table I. *Substrate(s) Requirement for Cyanide-Stimulated Protein Synthesis in Castor Bean Mitochondria*

The incubation conditions for the *in vitro* translations were as described in "Materials and Methods." The concentrations of the various components were: 20 mM acetate, 5 mM succinate, 2.2 mM NADH, 2.8 mM ADP, 5 mM ATP, 1 mM KCN. The ATP regenerating system is described under "Materials and Methods." The values shown are averages derived from each set of determinations carried out in triplicate and variations were between ±5% of the average values. ND, not detected.

Substrate(s)	Protein Synthesis	
	−KCN	+KCN
	dpm × 10^{-3}/mg protein/1 h	
Acetate	ND	ND
NADH	ND	ND
ADP	ND	ND
NADH + ADP	1077	310
Succinate	ND	ND
Succinate + ADP	1445	256
NADH + succinate	ND	ND
NADH + succinate + ADP	843	207
ATP regenerating system	810	956
NADH + ATP regenerating system	445	936
Succinate + ATP regenerating system	745	1920
ATP	432	512
ATP + succinate	780	1755

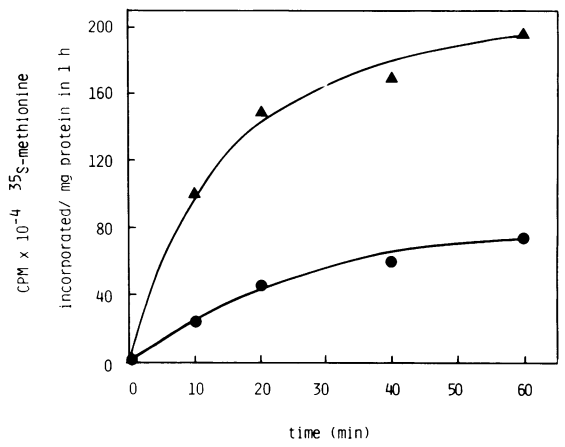

Figure 1. Time-course of mitochondrial protein synthesis. Organelle protein synthesis was carried out with 5 mM succinate and 5 mM ATP as fully described in "Materials and Methods" in the absence (●) or in the presence (▲) of 1 mM cyanide.

can be rationalized easily in terms of cyanide interacting with Cyt a_3 (21).

Interestingly, cyanide introduced a dramatic stimulation of protein synthesis when the externally added ATP was supplemented with either succinate or NADH; a greater than two-fold stimulation in protein synthesis was observed. The effect was more pronounced with succinate (Table I).

The time-course of incorporation of radiolabel into organelle synthesized proteins in the absence or presence of cyanide is shown in Figure 1. As shown, the stimulatory effect of cyanide was due to increased rate of protein synthesis. Cyanide does not appear to affect the duration of the reaction.

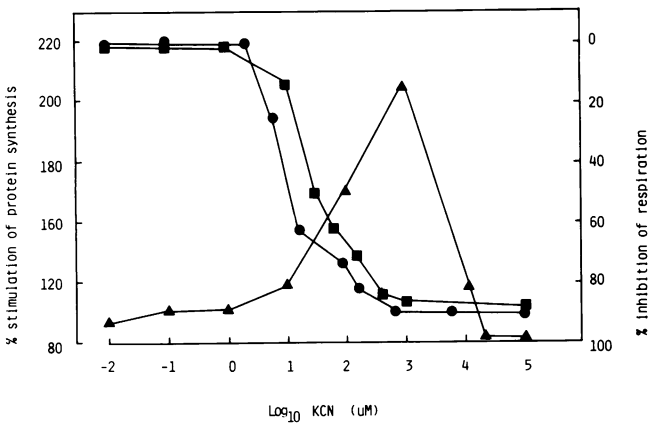

Figure 2. Effects of varying cyanide concentration on protein synthesis and respiration of the isolated castor bean mitochondria. Protein synthesis (▲) was performed in the presence of 5 mM succinate and 5 mM ATP. Increasing amounts of cyanide, dissolved in water, were added in a 5 μL volume to a final translation mixture volume of 50 μL. The protein synthetic activity was expressed as the percentage of control without cyanide. The average control value was 710×10^3 cpm [^{35}S]methionine incorporated into TCA insoluble protein/mg of mitochondrial protein in 1 h. Respiration rates were measured polarographically as described in "Materials and Methods" with either 10 mM succinate (■) or 5 mM NADH (●) and 0.25 mM ADP. The values shown are expressed as percentage of those obtained in the absence of cyanide. The control oxidation rates with succinate and NADH were 70 ± 6 and 48 ± 5 nmol O_2 consumed/min/mg of mitochondrial protein, respectively.

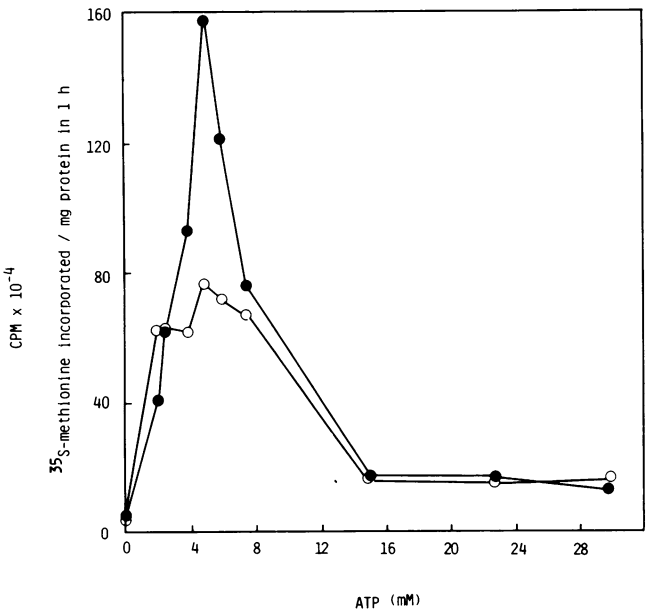

Figure 3. Effect of varying ATP on cyanide-stimulated mitochondrial protein synthesis. Protein synthesis was performed as described in "Materials and Methods" in the presence of 5mM succinate and with varying amounts of ATP in the absence (○) or in the presence (●) of 1 mM KCN.

The data illustrated in Figure 2 show the effect of varying cyanide concentration on mitochondrial protein synthesis measured in the presence of succinate and ATP. It can be seen that, as the concentration of cyanide was increased,

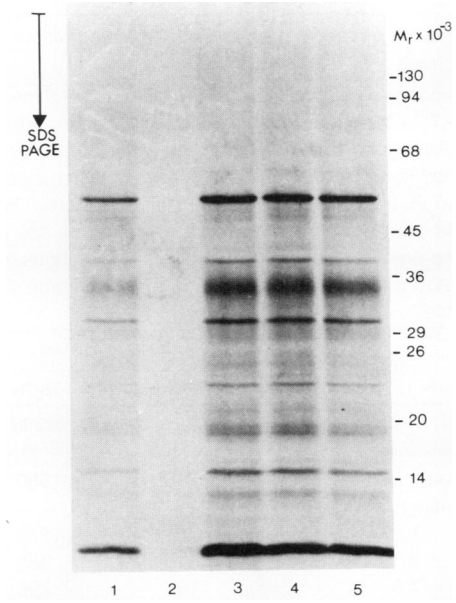

Figure 4. Effect of cyanide on polypeptides synthesized by isolated castor bean mitochondria. The experimental conditions for translations were as described in the legend in Figure 2. After incubation for 1 h, 10 μL of each translation mixture was mixed with two volumes of SDS sample buffer (11) and boiled for 3 min, after which the samples were electrophoresed on a gradient polyacrylamide gel (20–10% polyacrylamide). The fluorogram was exposed for 10 d. Track 1, 0 μM KCN; track 5, 25 μM KCN; track 4, 250 μM KCN; track 3, 2.5 mM KCN, track 2, 25 mM KCN. The positions of the mol wt markers (Sigma Chemicals, UK), traced from the dried Coomassie blue stained gel, are: myosin head, 200,000; β-galactosidase, 130,000; phosphorylase *a*, 94,000; BSA, 68,000; ovalbumin, 45,000; glyceraldehyde dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin inhibitor, 20,100; α-lactalbumin 14,200.

protein synthesis was progressively enhanced, reaching a peak at 1 mM cyanide. At higher concentrations of cyanide there was a rapid decline in protein synthesis. In contrast to the stimulatory effect of cyanide on mitochondrial protein synthesis, increasing concentrations of cyanide resulted in a progressive inhibition of the respiratory activity of the organelles, with the substrate, either succinate or NADH, supplemented with ADP. The maximum inhibition, 90%, of respiration was reached at 1 mM cyanide. The stimulation of protein synthesis by cyanide is greatest at the concentration of cyanide causing almost complete inhibition of respiration.

Both ATP and succinate (or NADH) were essential for the cyanide-stimulated castor bean mitochondrial protein synthesis. The effect of varying ATP concentration in the presence and absence of 1 mM cyanide is shown in Figure 3. The effect of increasing ATP concentration shows that protein synthesis is elevated with an optimum of around 5 mM ATP. However, in the presence of 1 mM cyanide, protein synthesis is more than doubled at the same concentration of ATP.

In order to explore whether the stimulatory effect of cyanide on castor bean mitochondrial protein synthesis was related to specific induction of new protein component(s), we analyzed the polypeptides synthesized in mitochondria treated with increasing concentrations of cyanide under the conditions which result in the stimulation of translation system. Figure

Table II. *Effect of Inhibitors on Cyanide-Stimulated Mitochondrial Protein Synthesis*

The translations were carried out in the presence of 5 mM succinate and 5 mM ATP with other conditions as described in "Materials and Methods." Antimycin, SHAM, oligomycin, and CCCP, prepared at predetermined concentrations in ethanol, each were added in 2 μL volume to 50 μL final volume of the translation mixture. The controls were treated with ethanol at final concentration of 4% (v/v). The values shown are averages of each set of determinations carried out in triplicate and the variations were ±7% of the average values. ND, not detectable.

Inhibitor	Protein Synthesis	
	−KCN	+KCN
	dpm × 10^{-3}/*mg protein*/*1 h*	
None	710	1560
Antimycin A (μM)		
1.87	781	1176
3.75	615	985
7.5	161	798
15	126	614
SHAM (μM)		
80	1227	863
400	1463	796
800	829	483
Oligomycin (μM)		
10	647	515
100	387	168
500	139	136
CCCP (μM)		
10	147	294
100	96	167
200	22	138
500	ND	103

4 shows the fluorographic profile of mitochondrial proteins after electrophoretic separation with equivalent amounts of protein loaded in each channel. The results show that the cyanide-stimulated mitochondrial protein synthesis results from increased incorporation of the radiolabel into all the newly synthesized proteins with no apparent quantitative change in the profiles.

The data presented in Table II show the effects on protein synthesis of antimycin A, oligomycin, CCCP[2] and SHAM, compounds which perturb energy conserving systems in the plant mitochondria. Antimycin A inhibits electron transport between Cyt *b* and c_1 (3, 7), oligomycin inhibits translocation of protons through the H^+ -ATPase (20), CCCP effectively deenergises the membrane by facilitating the movement of protons in response to the proton-motive force (8) and SHAM inhibits cyanide-insensitive alternative oxidase (1, 18, 19).

In the absence of cyanide, increasing concentrations of antimycin A, oligomycin and CCCP lead to gradual diminution of protein synthesis; results which could be anticipated from their known sites of action. The stimulatory effects of SHAM can be rationalized in terms of an inhibition of an energy leak via an alternative oxidase (14). In the presence of cyanide there are significant differences in the effects of oligomycin and CCCP. This indicates that the energy conserving H^+ -ATPase and the transmembrane proton-motive force are just as essential for protein synthesis in the presence of cyanide as in its absence. The stimulatory effects of cyanide on protein synthesis are also seen in the presence of antimycin A. Thus the site of action of cyanide in the stimulation of protein synthesis is not at the Cyt a_3 in the complex IV. More significantly, there is no stimulation by cyanide in the presence of SHAM. This strongly suggests that the alternative oxidase, in some way, plays a role in the mechanism of cyanide-stimulation of protein synthesis in these mitochondria.

The mode by which cyanide stimulates castor bean mitochondrial protein synthesis remains elusive. A plausible explanation is that cyanide in the presence of succinate (or NADH) may be facilitating more efficient utilization of external ATP. We are investigating the nature of this link between the alternative oxidase and the intra-organellar protein synthesising machinery. It also remains to be established whether the stimulatory effect of cyanide on mitochondrial protein synthesis is a phenomenon that is common among other higher plants.

[2] Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; SHAM, salicylhydroxamic acid.

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